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Fusion-Defective Mutants of Mouse Hepatitis Virus A59 Contain a Mutation in the Spike Protein Cleavage Signal

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Infection of primary mouse glial cell cultures with mouse hepatitis virus strain A59 results in a productive, persistent infection, but without any obvious cytopathic effect. Mutant viruses isolated from infected glial cultures 16 to 18 weeks postinfection replicate with kinetics similar to those of wild-type virus but produce small plaques on fibroblasts and cause only minimal levels of cell-to-cell fusion under conditions in which wild type causes nearly complete cell fusion. However, since extensive fusion is present in mutant-infected cells at late times postinfection, the defect is actually a delay in kinetics rather than an absolute block in activity. Addition of trypsin to mutant-infected fibroblast cultures enhanced cell fusion a small (two- to fivefold) but significant degree, indicating that the defect could be due to a lack of cleavage of the viral spike (fusion) protein. Sequencing of portions of the spike genes of six fusion-defective mutants revealed that all contained the same single nucleotide mutation resulting in a substitution of aspartic acid for histidine in the spike cleavage signal. Mutant virions contained only the 180-kDa form of spike protein, suggesting that this mutation prevented the normal proteolytic cleavage of the 180-kDa protein into the 90-kDa subunits. Examination of revertants of the mutants supports this hypothesis. Acquisition of fusion competence correlates with the replacement of the negatively charged aspartic acid with either the wild-type histidine or a nonpolar amino acid and the restoration of spike protein cleavage. These data confirm and extend previous reports concluding cleavage of S is required for efficient cell-cell fusion by mouse hepatitis virus but not for virus-cell fusion (infectivity).

Mouse hepatitis virus (MHV), a member of the family *Coronaviridae*, is a common pathogen of mice. MHV strain A59 (MHV-A59) is a positive-stranded enveloped virus with an approximately 31-kb genome RNA. Three structural proteins have been identified in MHV-A59 (21). The nucleocapsid protein (N) is thought to bind to the RNA and form the helical nucleocapsid (23). The membrane protein (M) is an integral membrane glycoprotein (1) that may direct the assembly of virions in the Golgi complex (18). The peplomer or spike protein (S) is the glycoprotein present on the surface of the virion and is responsible for binding to the cellular receptor (3). Consequently, antibodies directed at specific epitopes on S are capable of neutralizing viral infectivity. This protein is translated as a 145-kDa protein and core glycosylated in the endoplasmic reticulum. Following transport to the Golgi complex, the carbohydrate moieties are modified, giving a molecular mass of approximately 180 kDa. Subsequently, the spike is cleaved into two 90-kDa fragments termed S1 (N terminal) and S2 (C terminal). Some of the S protein not assembled into virions is transported by the secretory system to the cell surface where it is free to interact with adjacent cells, resulting in cell fusion (syncytia) (21). Expression of the coronavirus S protein in the absence of infection (via vaccinia virus vectors) and other viral proteins is sufficient to induce efficient cell fusion (22, 25, 26).

Cleavage of the spike occurs in many but not all coronaviruses, and in those strains in which cleavage is not observed (e.g., feline infectious peritonitis virus, porcine transmissible gastroenteritis virus), cell fusion occurs normally. However, in MHV, cell fusion correlates with the level of cleavage; consequently, the processed (cleaved) protein rather than the precursor is believed to be fusogenic (5). Proteolytic cleavage of the coronavirus spike protein occurs adjacent to a sequence of basic amino acids on the C terminus of S1 (15). This motif is conserved among MHV strains and in avian infectious bronchitis virus. In paramyxoviruses (8), many retroviruses (17), and influenza A viruses (2), all of which contain envelope proteins that are posttranslationally cleaved, analogous stretches of basic amino acids occur adjacent to the site of cleavage.

We previously reported that infection of primary mouse glial cells with MHV-A59 results in a productive but persistent infection (9, 14). These infections occur in the absence of any clearly discernible cytopathic effect. Because MHV can persist in the murine central nervous system and cause chronic demyelination, we were interested in identifying changes in the virus during persistence in glial cells. In this report, we present data showing that during persistence in glial cultures, small-plaque, fusion-defective mutants arise. Sequencing of portions of the gene encoding the spike protein revealed that six mutants derived from two independent cultures contained the same histidine-to-aspartic acid substitution in the cleavage signal region. Analysis of virion structural proteins suggests that this mutation prevents proper proteolytic cleavage of the 180-kDa spike precursor. Evidence that the defect in cleavage is responsible for the fusion deficiency seen in infected fibroblasts comes from studies of revertants in which fusion, cleavage of the spike, and loss of the acidic aspartic acid residue coincide.

MATERIALS AND METHODS

Virus and cells. MHV-A59, obtained from Lawrence Sturman (Albany, N.Y.), was propagated in mouse L2 cells. The virus stock had been grown previously at 40°C to eliminate any temperature-sensitive mutants that might be present.

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Primary mixed glial cell cultures were made from dissociated brains of newborn C57BL/6 mice essentially as described previously (9, 14) and were used 10 to 15 days after plating. These cultures were 90 to 95% astrocytes as determined by positive immunostaining for glial fibrillary acidic protein (data not shown). Primary glial cultures, L2 cells, and 17Cl-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

Infection of glial cells and isolation of virus. Glial cell cultures were infected with MHV-A59 at a multiplicity of infection (MOI) of 5 and maintained at 37°C as previously described (14). Three independent cultures were established and are referred to as the A, B, and C cultures. The medium was removed twice weekly, and the cells were refed with fresh medium. Medium taken from infected cultures and containing virus was stored at −80°C for virus titration and isolation. Infectious virus was quantitated by plaque titration (see below). Virus was isolated from the medium by three plaque-to-plaque purifications and then grown to high titer on L2 cells at 32°C.

Virus titrations. Plaque titration of virus was done on L2 cells. Briefly, virus was diluted serially in gelatin saline (19) and inoculated onto cell monolayers. After a 1-h incubation at 32°C, the cells were overlaid with DMEM containing 2% FBS and 0.7% agarose and placed at 37°C for 24 h. Cells were stained by overlaying the agarose plug with DMEM containing 2% FBS, 0.7% agarose, and 0.02% (wt/vol) neutral red. In some cases, titrations were done at 39°C (nonpermissive temperature) or at 32°C (permissive temperature) to identify and quantitate temperature-sensitive mutants. When assays were done at 32°C, the infected monolayers were incubated for 48 h before the addition of neutral red.

Titration of virus in the presence of 30 μg of trypsin (Sigma) per ml was done as described above except that cell monolayers were washed twice in Tris-buffered saline prior to infection and FBS was omitted from the medium.

Viral growth kinetics. L2 cell monolayers were prepared in 2-dram (7.4-ml) screw-cap vials (Wheaton) in DMEM with 10% FBS. Confluent monolayers were infected at an MOI of 5 and incubated for 1 h at 37°C. Following adsorption, the cells were washed three times with Tris-buffered saline and then fed with 1.0 ml of DMEM–10% FBS. Vials were removed at regular intervals and stored at −80°C for titration of virus.

Immunoprecipitation of spike protein. L2 or 17Cl-1 cells were infected with 5 PFU of wild-type A59, fusion-defective mutants, or fusion-competent revertants per cell. At 5 h postinfection, the medium was removed and the cells were refed with DMEM lacking serum and methionine. After a 30-min incubation at 37°C, the infected cells were labelled with 50 μCi of [^{35}S]-TransLabel (ICN) per ml in methionine-free DMEM for 6 h. Virus in the medium was collected by centrifugation in an SW41 rotor at 32,000 rpm for 90 min. The pellets were lysed in RIPA buffer (50 mM Tris HCl [pH 7.5], 100 mM NaCl, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 1% [vol/vol] Nonidet P-40, 1% [wt/vol] sodium deoxycholate, 100 μg of phenylmethylsulfonyl fluoride per ml). The spike protein was immunoprecipitated with a monospecific polyclonal goat serum (AO4) prepared against the spike protein (kindly provided by K. Holmes, Bethesda, Md.), and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Fusion assay. L2 cells were infected with virus at an MOI of 5. Following adsorption, the cells were fed with DMEM containing 10% FBS and incubated at 37°C. The proportion of nuclei contained in syncytia was determined as a function of time after infection with either ethanol-fixed crystal violet-stained cells or photographs of infected cells. In both methods, three random fields were counted and the percentage of nuclei contained in syncytia was determined.

To assess the effect of trypsin on cell fusion, we infected the cells as above except that serum was omitted from the growth medium. At selected times, the cells were washed three times with phosphate-buffered saline (PBS) and then treated for 30 min at 37°C with 2.5 or 5 μg of trypsin per ml of PBS. Afterward, the trypsin was removed, and the cells were washed once and then fed with DMEM containing 10% FBS. Fusion was determined after a 90-min incubation at 37°C and compared with fusion in cells incubated in the absence of trypsin by Student's *t* test.

RNA sequencing. Sequence information was obtained both by direct sequencing of viral RNA and by sequencing of polymerase chain reaction (PCR)-amplified cDNA derived from reverse transcription of viral RNA.

The cleavage signal region of wild-type A59 and the fusion-defective mutants was determined by direct RNA sequencing of cytoplasmic RNA (50 to 70 μg) with reverse transcriptase and dideoxynucleotides (Boehringer Mannheim). Sequencing products were treated with 10 to 20 U of terminal transferase (Boehringer Mannheim) to avoid ambiguities due to strong stops. Primer CSN (5′-CATCGGAGT GTATGGCTC-3′), complementary to sequence approximately 30 nucleotides downstream of the cleavage signal region (nucleotides 2185 to 2202 in the sequence of Luytjes et al. [15]), was purchased from Operon Technologies.

The entire S gene sequence of wild-type and mutant viruses was determined from DNA fragments obtained by reverse transcription-PCR. Cytoplasmic RNA was reverse transcribed with random hexamers (Boehringer Mannheim) and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). PCR amplification of the cDNA templates was done with *Taq* polymerase (Boehringer Mannheim) and six pairs of primers (obtained from K. Holmes) yielding six overlapping fragments approximately 700 to 800 nucleotides in length spanning the entire S gene. PCR products were purified with Promega's Magic PCR Prep and then sequenced with the fmol DNA sequencing kit from Promega following the manufacturer's recommended protocol with the extension termination reaction and ^{35}S-dATP.

Immunofluorescence. L2 cells grown on 13-mm coverslips were infected in duplicate with wild-type A59 or the fusion-defective mutants at a multiplicity of 1. At selected times postinfection, the cells were washed with PBS, and one coverslip of each pair was permeabilized with methanol for 5 min. The cells were incubated for 60 min at 37°C with goat antispike serum (AO4) diluted in DMEM containing 10% FBS and 0.02% (wt/vol) sodium azide. After three washes with PBS, fluorescein isothiocyanate-conjugated rabbit anti-goat F(ab′)$_2$ (Cappel) diluted as above was added and incubated at 37°C. The cells were washed with PBS and mounted onto glass slides with 90% glycerol in PBS containing 25 mg of 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) per ml.

RESULTS

Isolation of mutants from persistently infected primary glial cells. We reported previously (9, 14) that unlike infection of murine fibroblasts with MHV-A59, infection of primary glial cell cultures causes a productive but persistent infection with minimal cytopathic effect. To better understand virus-glial cell interactions, we have begun to examine the evolu-

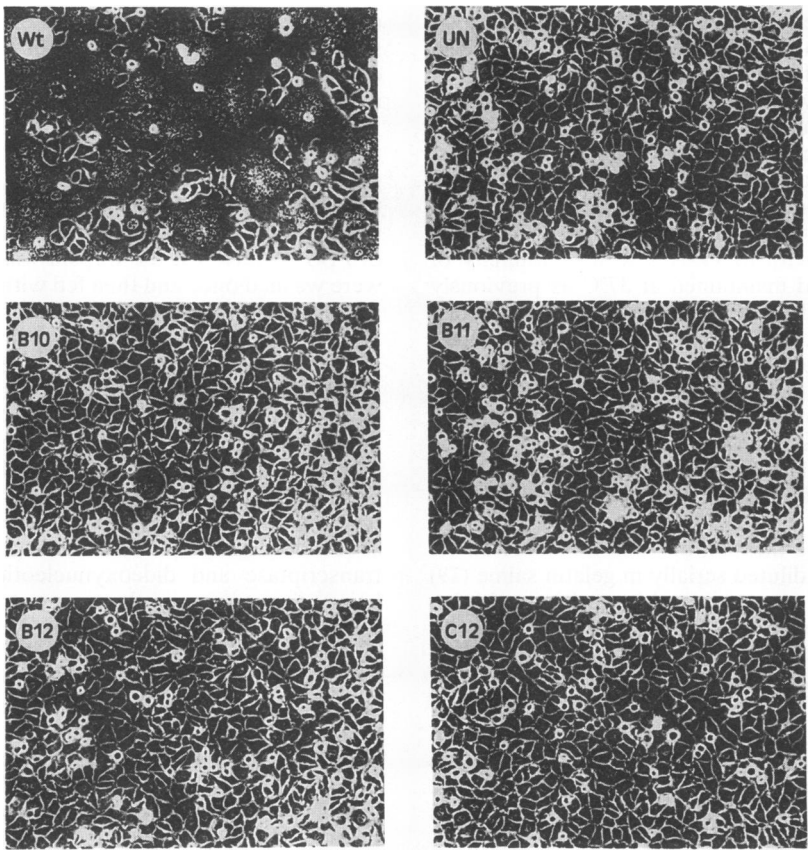

FIG. 1. Viruses isolated from persistently infected glial cell cultures are defective in cell fusion. L2 cells were infected at 37°C with 5 PFU of virus per cell and photographed at 10 h postinfection. Shown are cells infected with wild-type A59 (Wt), mutants B10, B11, B12, and C12, and mock-infected cells (UN).

tion of MHV during long-term infection in these cultures. We infected three separate flasks of glial cells with A59 at high multiplicity. At weekly intervals, the medium was removed from the cultures and stored at −80°C for titrations, and the cells were refed with fresh medium.

Titers of infectious virus in the medium were determined by plaque assay. All three cultures produced virus continuously over the 16- to 18-week period, with levels of virus varying from 10^6 to 10^7 PFU per culture (data not shown). These titrations also showed a change in plaque morphology of the virus from two of these cultures (B and C cultures). The wild-type virus used to initiate the infections produces plaques 2 to 3 mm in diameter with a highly lytic morphology (data not shown). This morphology predominated through the first 8 to 12 weeks of infection. Beginning around week 12 postinfection, smaller and less lytic plaques began to appear, and by 16 to 18 weeks postinfection, all plaques exhibited this morphology.

Medium was taken from cultures at 1, 6, 12, and 18 weeks postinfection (16 weeks for culture C). Three clones were isolated by three plaque-to-plaque purifications from each culture at each time point. Plaque-purified virus isolated from the earlier times postinfection, for example, clones B4 and B6 (6 weeks postinfection) and clone B7 (12 weeks postinfection), displayed a wild-type plaque morphology. Virus isolated at 16 to 18 weeks postinfection from cultures B and C retained the altered plaque morphology initially seen in the uncloned population. All clones from culture A produced plaques that were more lytic than those of the B and C clones and will not be described further here.

Fusion phenotype of the mutants. Infection of L2 cells with the plaque-purified viruses isolated from the B and C cultures after 16 to 18 weeks of infection showed markedly delayed fusion kinetics. At 10 h postinfection wild-type A59 caused nearly complete fusion of the cell monolayer, while cell fusion in mutant-infected cultures involved less than 10% of the cells (Fig. 1 and 2). However, by 28 h postinfection, all mutants but C10 and C11 induced fusion at levels similar to those of the wild type. Clones C10 and C11 fused cells at a slower rate than the other four mutants, and by 28 h postinfection, less than 40% of the cells had fused (Fig. 2).

The small-plaque phenotype and the delayed kinetics of fusion suggested that the mutants replicated more slowly or grew to lower titers than wild-type A59. Therefore, we examined the growth kinetics of the mutants by infecting L2 cells at 37°C and measuring the virus yield by plaque titration. Four mutants (B10, B11, B12, and C12) replicated with kinetics virtually identical to those of the wild type and achieved similar titers (Fig. 3). The growth kinetics of mutants C10 and C11 were delayed, but by 24 h postinfection, the yield of virus was comparable to that of the wild type. Thus, at least for mutants B10, B11, B12, and C12, the delay in cell fusion was not due to slow growth kinetics or reduced virus yields.

Gallagher et al. (6) reported previously that fusion-defective mutants of MHV-4 were able to cause cell fusion only

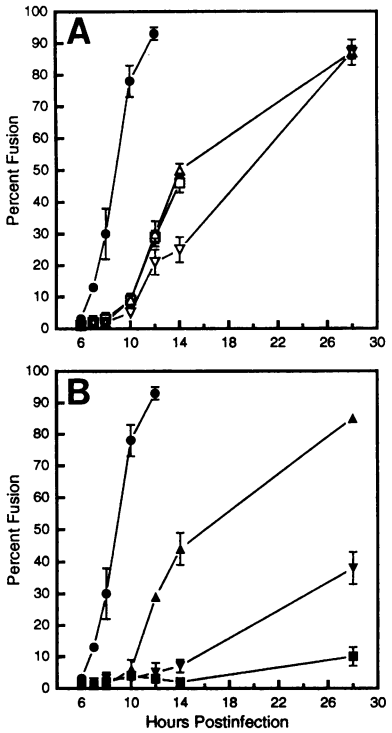

FIG. 2. Fusion in mutant-infected cells is delayed. L2 cells were infected as described in the legend to Fig. 1. At the indicated times postinfection, the cells were photographed and the mean percentage of nuclei in syncytia was plotted. Error bars represent the standard deviation of the mean determined from three random fields. (A) Wild-type A59 (●) and mutants B10 (▽), B11 (□), and B12 (△). (B) Wild-type A59 (●) and mutants C10 (▼), C11 (■), and C12 (▲). Sloughing of syncytia in wild-type samples at 14 and 28 h postinfection and in B11 samples at 28 h postinfection prevented quantitation of those samples.

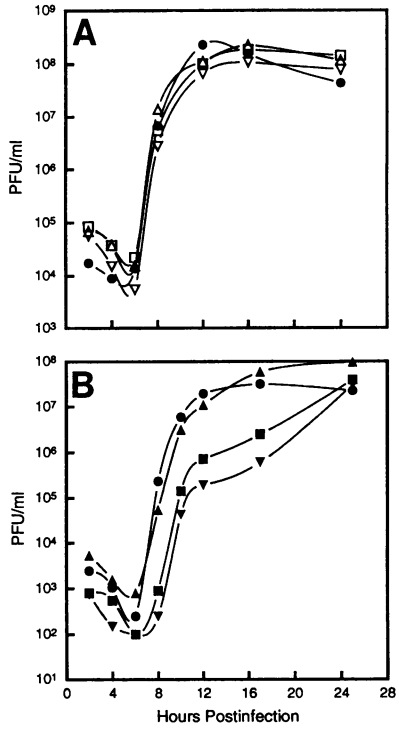

FIG. 3. Growth kinetics of wild-type A59 and the fusion-defective mutants in L2 cells. Cells were infected at 37°C at an MOI of 5. The yield of infectious virus at the indicated times postinfection was determined by plaque titration. (A) Wild-type A59 (●) and mutants B10 (▽), B11 (□), and B12 (△). (B) Wild-type A59 (●) and mutants C10 (▼), C11 (■), and C12 (▲).

under acidic conditions but not at neutral or slightly alkaline pHs. To determine whether the mutants isolated from the B and C cultures late after infection also were dependent on an acidic pH for fusion, cells infected with either wild-type or mutant viruses were treated for 30 min at various times postinfection with medium or Tris-saline at pHs ranging from 5 to 9. After an additional 90 min of incubation in the presence of normal DMEM–10% FBS, the cells were examined for fusion. The mutants maintained their fusion-defective phenotype regardless of pH (data not shown), suggesting that these viruses were fundamentally different from the MHV-4 mutants described by Gallagher et al. (6).

Cell surface expression of spike protein. Fusion of cells during replication (i.e., fusion from within) is mediated by the spike protein on the surface of infected cells. Failure of the spike protein to be transported to the cell surface would account for the lack of fusion in mutant-infected cells. To test this, cells were infected at low multiplicity with wild type and the mutants and stained for either cytoplasmic or surface-bound spike protein by immunofluorescence at various times between 5 and 9 h postinfection. As shown in Fig. 4, at 8 h postinfection, the spike protein can be readily detected on the surface of wild type- and B11-infected cells. Examination of the surface expression of S at times between 5 and 9 h postinfection suggests that the kinetics of transport of mutant S to the cell surface is similar to that of wild-type S. Similar results were obtained with the other mutants isolated from the B and C cultures 16 to 18 weeks after infection.

Spike protein cleavage defect. Since fusion of cells during infection is directly correlated with the extent of cleavage of the spike protein (5), we examined the possibility that the mutants were defective in the cleavage of the 180-kDa spike precursor to the 90-kDa S1 and S2 subunits. Cells were infected with either wild type or mutants at high multiplicity, and at 7 h postinfection, the cells were washed and incubated for 30 min in 2.5 or 5.0 μg of trypsin per ml. After the trypsin pulse, the infected cultures again were washed, medium containing 10% serum was added, and the incubation was continued for an additional 90 min. Cells infected with all mutants except C10 and C11 exhibited higher levels of fusion when treated with trypsin than in control cultures (Fig. 5). However, the increase in fusion seen in the presence of 5 μg of trypsin per ml, although statistically significant ($P < 0.05$), was generally fivefold or less, and never more than 30% of the cells fused.

Similar experiments in which trypsin was incorporated into plaque assays supported the hypothesis that the fusion phenotype of the mutants was due to a proteolytic processing defect. In the absence of trypsin, the fusion mutants made plaques on L cells that were small and nonlytic. In contrast, in the presence of 30 μg trypsin per ml, the mutants made large, lytic plaques that were nearly identical to those of wild-type A59 (data not shown).

Together, these data suggested that trypsin was able to at least partially restore the fusion phenotype of the mutants. Because of the correlation between cleavage of S and fusion

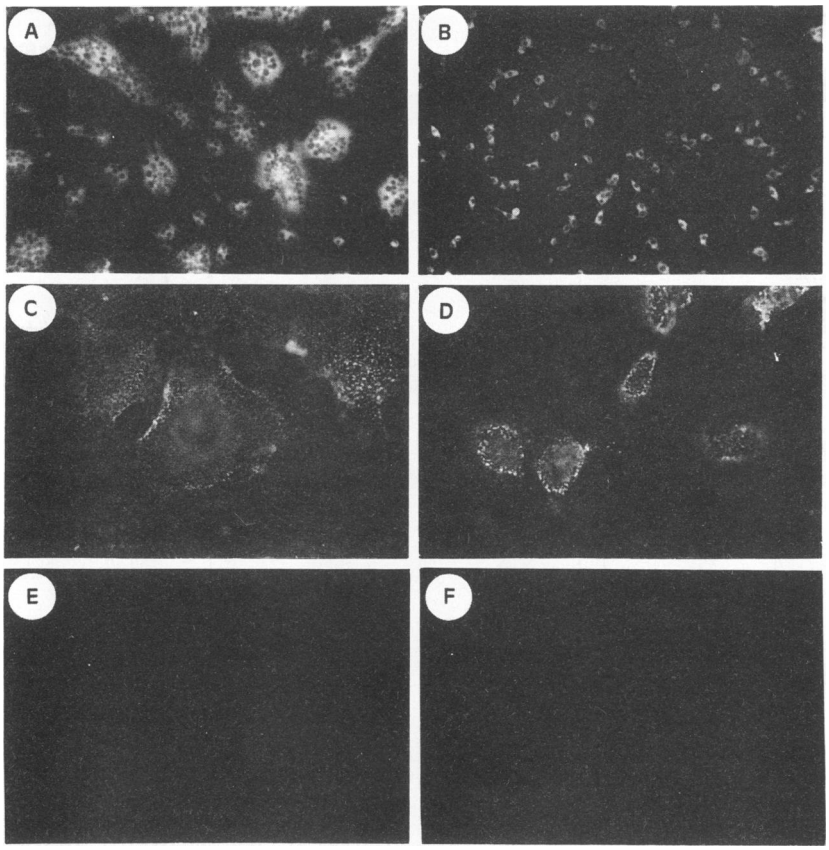

FIG. 4. Spike protein is expressed on the cell surface in mutant-infected L2 cells. Cells were infected with 1 PFU of wild-type A59 (A, C, and E) or mutant B11 (B and D) per cell or were mock infected (F). At 8 h postinfection, the cells were permeabilized with methanol (A, B, E, and F) or left untreated (C and D) and incubated with antiserum AO4 (goat antispike; A, B, C, D, and F) or normal goat serum (E) followed by fluorescein isothiocyanate-conjugated rabbit anti-goat $F(ab')_2$ fragments. Cytoplasmic fluorescence (A, B, E, and F) was photographed at ×100 and surface fluorescence (C and D) at ×400.

(5), it seemed likely that the trypsin was cleaving the spike precursor into its active subunits. To test this directly, we examined the amount of cleaved and uncleaved S protein in both wild-type and mutant virions. We examined the spike in virions because only a small fraction of S in the infected cell is cleaved. Cells infected with wild type or the mutants were labeled with [^{35}S]-TransLabel from 6 to 12 h postinfection, and the virions were pelleted from the medium by centrifugation. After lysis in radioimmunoprecipitation assay (RIPA) buffer, the spike protein was immunoprecipitated and analyzed by SDS-PAGE. The spike protein in wild-type A59 was present in both the 180-kDa and the 90-kDa forms (Fig. 6). As previously reported by Frana et al. (5), the spike protein from wild-type virions was only partially cleaved. Mutant virions, in contrast, contained only the uncleaved 180-kDa spike precursor. These results suggest that the fusion-defective phenotype of the mutants is a direct result of the inability of the spike protein to be processed into its mature form.

To better understand the nature of the defect in these mutants, we directly sequenced the RNA encoding the S1/S2 junction in each of the clones. The sequence obtained for wild-type A59 was identical to that previously published by Luytjes et al. (15). As shown in Fig. 7, directly upstream from the S2 amino terminus is the highly basic amino acid sequence Arg-Arg-Ala-His-Arg that is thought to act as a signal for cleavage by what is presumed to be a cellular protease. Sequencing of the S1/S2 junction in the mutants showed that all three B mutants as well as each of the C mutants contained the same C-to-G point mutation in codon 716. This mutation would substitute a negatively charged aspartic acid for the weakly basic histidine in the cleavage signal. We suggest that the introduction of a negatively charged amino acid into this highly basic region destroys the signal or otherwise prevents its usage and thereby prevents cleavage of the spike.

Since the mutants could contain additional mutations in the spike protein, we sequenced the entire S genes of the wild type and mutants B11 and C12. This was done by PCR-mediated sequencing of cDNAs derived from infected-cell RNA. We chose to sequence the RNA rather than cloned DNA fragments in order to obtain a consensus sequence for the viral genome RNA rather than the sequence of a cloned cDNA. We found that wild-type S varied by two nucleotides from the sequence for MHV-A59 published by Luytjes et al. (15). One of these mutations, an A-to-G change at nucleotide 3045, was silent, while the other, a G-to-A change at nucleotide 293, resulted in the substitution of a serine for an asparagine. These sequence differences were also observed by Cynthia Ricard in the laboratory of Lawrence Sturman (23a). The B11 and C12 mutant spike genes were identical to wild type with the exception of the cleavage signal mutations described above and an additional mutation at nucleotide 476 (codon 159) resulting in a glu-

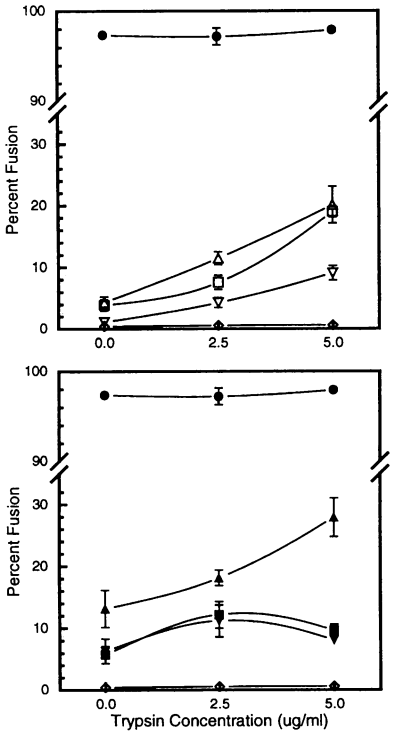

FIG. 5. Trypsin enhances cell fusion in mutant-infected cells. L2 cells were infected with 5 PFU of wild-type A59 (●) or mutants from the B (B10 [▽], B11 [□], B12 [△] or C (C10 [▼], C11 [■], C12 [▲]) cultures per cell or were mock infected (◇). At 7 h postinfection, the cells were washed and treated with the indicated concentrations of trypsin for 30 min at 37°C. After the trypsin was removed, the cells were washed with PBS and then incubated in DMEM–10% FBS at 37°C for 90 min. The cells were stained with 1% crystal violet in 70% methanol, and the percentage of nuclei in syncytia was determined. Plotted are the mean and standard deviation determined from three random fields.

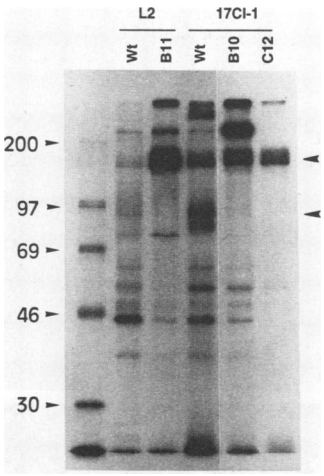

FIG. 6. Spike protein in fusion-defective mutants is not cleaved. L2 or 17Cl-1 cells were infected with the indicated viruses (wt, wild type) and labelled with [^{35}S]-TransLabel as described in Materials and Methods. Virus in the medium was pelleted, and the spike protein was immunoprecipitated with antiserum AO4 (goat antispike). Proteins were analyzed by SDS-PAGE. Molecular mass markers (in kilodaltons) are shown on the left. Bands corresponding to the uncleaved spike precursor (180 kDa) and the cleaved subunits (90 kDa) are indicated with arrowheads on the right.

tamine (Q)-to-leucine (L) substitution in the amino-terminal portion of S1. As described below, analysis of revertant viruses suggests that the Q-to-L mutation is not linked to the defective fusion phenotype.

Fusion-competent revertants. To test the correlation between fusion competence, cleavage of S, and the sequence in the putative cleavage signal, we isolated fusion-competent revertants of several mutants. These revertants were isolated following serial low-multiplicity passages in L2 cells and were chosen based on their wild-type plaque morphology. Infection of cells with these viruses induces cytopathic effect similar to that of wild-type A59 (Fig. 8). For example, at 10 h postinfection, when mutant-infected monolayers have only a low level of syncytia (Fig. 1 and 2), cells infected with wild type or revertants C11R1 or C12R1 are nearly completely fused. In contrast, B11S1, a subclone of B11 that was isolated following serial passage but retained the mutant plaque phenotype, did not revert in fusion phenotype (Fig. 8). When the spike protein in revertant virions was examined as described previously for mutant virions, we found that all revertants examined contained the cleaved form of S (Fig. 9, Table 1).

Sequence analysis of the revertants in the region of the cleavage site revealed that restoration of cleavage was associated with the loss of the aspartic acid residue seen in the mutants (Table 1). One clone (C12R1) reverted to the wild-type histidine residue, but more often substitution of the aspartic acid with a small noncharged amino acid was found. Sequencing of B11S1 showed that the aspartic acid mutation was maintained in the cleavage signal, consistent with an association of the aspartic acid mutation with the fusion-defective phenotype (Table 1). The glutamine-to-leucine mutation present in the S1 portion of the mutant S genes was also present in all the revertants examined,

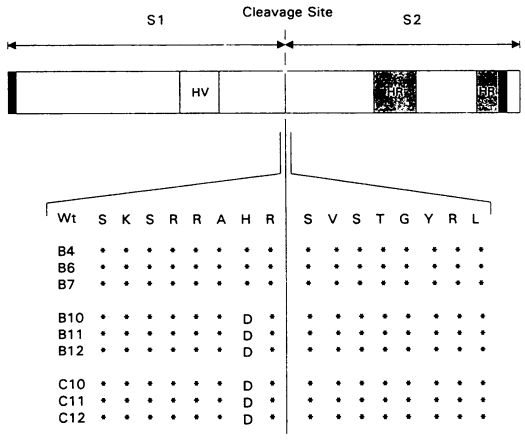

FIG. 7. Schematic of the spike protein and the nucleotide and deduced amino acid sequence for the S1/S2 junction in wild type (Wt) and the fusion-defective mutants. RNA from virus-infected cells was sequenced directly with primer CSN and reverse transcriptase. Clones B4 and B6 (isolated 6 weeks after infection) and clone B7 (isolated 12 weeks after infection) possess a wild-type fusion phenotype. Clones B10, B11, and B12 (isolated 18 weeks after infection) and clones C10, C11, and C12 (isolated 16 weeks after infection) are fusion defective. Solid bars, hydrophobic domains; HV, hypervariable region; HR, heptad repeats.

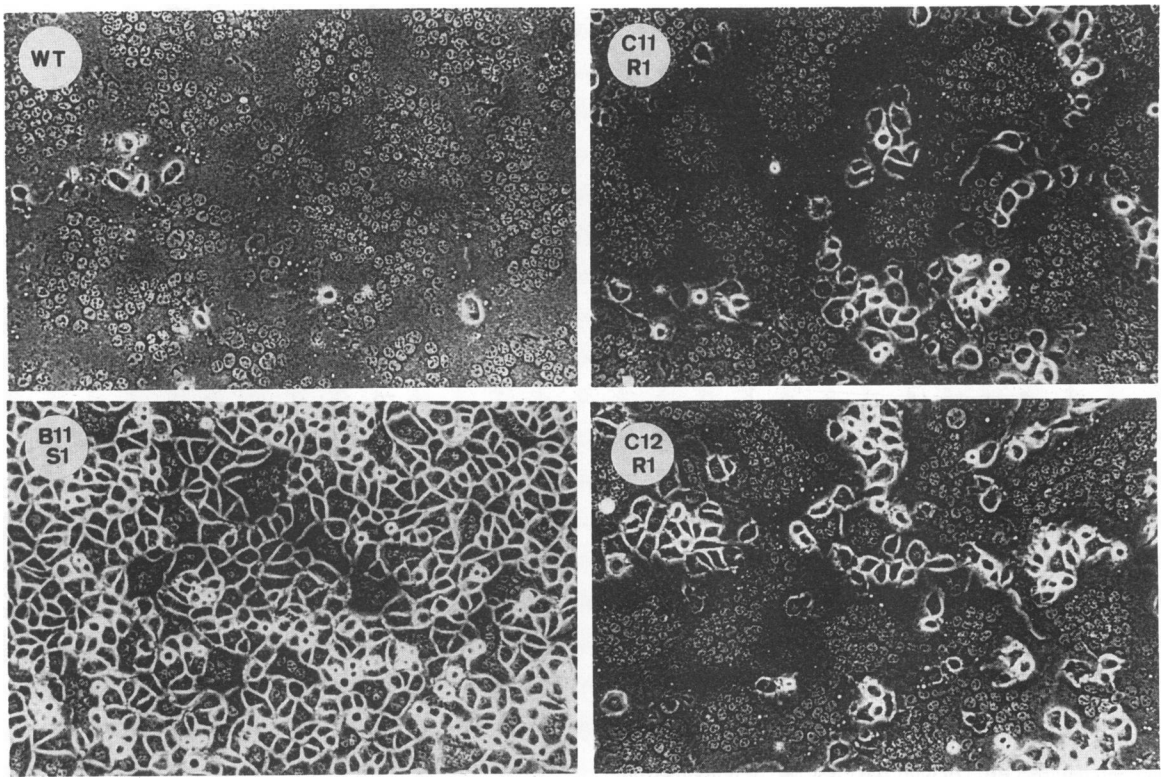

FIG. 8. Revertants of the fusion-defective mutants induce normal cell fusion. L2 cells infected at 37°C with 5 PFU of wild-type A59 (Wt) or revertants of C11 (C11R1) or C12 (C12R1) per cell show typical cell fusion at 10 h postinfection. In contrast, the fusion phenotype of clone B11S1, isolated following serial passage of clone B11, did not revert.

indicating that this mutation is not associated with the defective fusion phenotype. We conclude that efficient fusion requires cleavage of the spike protein and that drastic alterations of the cleavage signal, such as the incorporation of negatively charged amino acids, can prevent its recognition by the (cellular) protease.

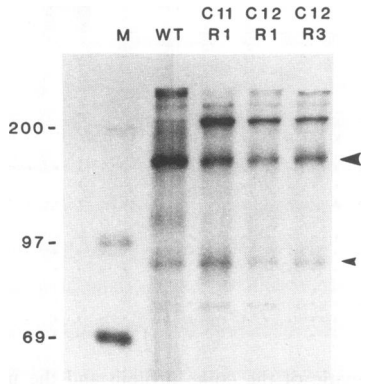

FIG. 9. Spike protein in fusion-competent revertants is cleaved. 17Cl-1 cells were infected with the indicated viruses and labelled with [^{35}S]-TransLabel as described in Materials and Methods. Virus in the medium was pelleted, and the spike protein was immunoprecipitated and analyzed as described in the legend to Fig. 6. Molecular mass markers (in kilodaltons) are shown on the left. Bands corresponding to the uncleaved spike precursor (180 kDa) and the cleaved subunits (90 kDa) are indicated with large and small arrowheads, respectively.

DISCUSSION

We report here the characterization of viruses isolated from persistent infections of murine glial cells. Most of these viruses display normal growth kinetics in murine fibroblasts and achieve titers similar to the parental A59 virus from which they were derived. However, they produce small, nonlytic plaques and fail to cause efficient fusion in infected cells. Two clones, C10 and C11, are somewhat different from the others. These mutants are temperature sensitive and replicate with slower kinetics than wild type at 37°C. How-

TABLE 1. Summary of MHV-A59 fusion mutants and revertants

Virus	Codon 159	Codon 716	Fusion[a]	Spike cleavage
Wild type	CAG (Q)	CAC (H)	+	+
B10	ND[b]	GAC (D)	−	−
B11	CTG (L)	GAC (D)	−	−
B12	CTG (L)	GAC (D)	−	−
C10	ND	GAC (D)	−	ND
C11	ND	GAC (D)	−	ND
C12	CTG (L)	GAC (D)	−	−
B11S1	ND	GAC (D)	−	ND
C11R1	ND	GCC (A)	+	+
C12R1	CTG (L)	CAC (H)	+	+
C12R2	CTG (L)	GCC (A)	+	ND
C12R3	CTG (L)	GGC (G)	+	+

[a] Cell fusion at 10 h postinfection.
[b] ND, not done.

ever, even though they achieve titers comparable to those of the other mutants, they do not induce the same level of fusion in cells, even if the assays are done at 32°C (the permissive temperature for C10 and C11). Although this suggests that the fusion defect is not due directly to the temperature-sensitive defect, the slow growth kinetics of C10 and C11 may indirectly affect the level of fusion in infected cells.

All six mutants examined contain a histidine-to-aspartic acid substitution within the basic amino acid cleavage signal thought to be responsible for correct proteolytic processing of the spike. (It is important to note that this mutation probably occurred only twice, once in each culture, rather than in each mutant individually.) Analysis of the spike protein present in virions shows that these fusion mutants do not contain detectable amounts of cleaved spike, but rather incorporate the uncleaved 180-kDa precursor. Restoration of fusion competence and cleavage of S in revertant viruses is associated with the replacement of the acidic aspartic acid residue with either the wild-type histidine or a noncharged amino acid. We suggest that incorporation of aspartic acid within the basic cleavage signal prevents cleavage by preventing either its recognition by or its interaction with the protease responsible for cleavage. Sawicki (20) isolated a small-plaque mutant of MHV-A59 from persistently infected 17Cl-1 cells that is similar to the mutants described here. Purified virions of this mutant contained only the uncleaved 180-kDa spike protein; furthermore, 100-fold more trypsin was required to convert the 180-kDa protein to the 90-kDa form. Interestingly, this mutant contains a substitution of asparagine for the histidine residue in the cleavage signal (20a).

The phenotypes of the mutants described here suggest that cleavage of S is a prerequisite for efficient cell-to-cell fusion. The levels of fusion seen at late times postinfection in mutant-infected cells may be due to low but undetectable levels of cleaved S that accumulates over time on the cell surface. Alternatively, the uncleaved spike protein might be capable of causing fusion, albeit inefficiently, and only when high levels of S accumulate on the cell surface is extensive fusion seen. Finally, it is possible that cleavage increases the affinity of the spike for the cellular receptor or otherwise alters their interaction and thus only indirectly affects fusion.

We have argued based on immunofluorescence data that the fusion defect is not due to a lack of expression of the spike protein on the cell surface. Since Holmes and Behnke (11) observed that virions released from MHV-infected cells could readsorb to the plasma membrane, it is possible that our immunofluorescence experiments were detecting virions rather than membrane-bound S. We consider this unlikely since surface-associated S can be observed as early as 5 to 6 h postinfection in cells infected with wild type or mutants, while infectious virus is not detectable until after 6 h postinfection. Therefore, at the earlier times after infection, it is unlikely that virion spike proteins are being detected.

Two other classes of fusion-defective mutants of MHV have been described previously. Gallagher et al. (6) showed that a mutant of MHV-4 recovered from a persistently infected neural cell line contained mutations within the heptad repeats of the S2 subunit of the spike protein. The heptad repeat region is believed to be important in the oligomerization of S protein (4). These mutations caused a change in the pH dependence of fusion such that an acid pH was required by the mutant to induce syncytia in infected cells. Gallagher et al. (7) also reported that neutralization-resistant mutants of MHV-4 selected with a monoclonal antibody to S were defective in their ability to induce syncytia. These mutants contain deletions in the hypervariable region of the S1 subunit (7, 16). A JHM isolate from the spinal cord of a demyelinated rat was also found to contain a deletion in the hypervariable region of S1 (13). While the mechanism by which any of these mutations effect fusion in these MHV-4 and JHM variants is unknown, the identification of these variants clearly demonstrates that different regions in the protein are crucial for proper function of the protein. We identified here a third region of S, the cleavage signal region, that appears to affect the ability of S to induce cell-to-cell fusion.

Recently, Stauber et al. (22) and Taguchi et al. (25) cloned the spike gene of MHV-JHM and introduced mutations in the cleavage signal that prevent cleavage of the spike protein. Transient expression of these cDNAs in a vaccinia virus system resulted in cell fusion (22, 24). However, in at least one case, the kinetics of fusion in cells expressing the mutant protein were delayed by 6 to 12 h (24). This is similar to the fusion phenotype exhibited by our A59 mutants. While minor differences exist between these in vitro fusion experiments and the fusion caused by our mutants, all the data suggest that cleavage enhances the ability of the spike to induce fusion but is not strictly required.

Proteolytic processing of envelope proteins is common in many viruses, and for some it is a requirement for cell-to-cell fusion as well as virus-cell fusion or infectivity. The fusion proteins of paramyxoviruses (8), orthomyxoviruses (2), and retroviruses (17) are cleaved into two subunits by cellular proteases. Similar to MHV, the site of cleavage is preceded by a group of basic amino acids. Glickman et al. (8) have proposed that for paramyxoviruses, a high content of basic amino acids at this site directly affects the efficiency with which the fusion protein precursor, F_0, is cleaved. Furthermore, the more highly basic cleavage signals are associated with increased virulence. The cleavage of the hemagglutinin of influenza virus is also influenced by the content of basic amino acids near the cleavage site (12) and, as for paramyxoviruses, is a major determinate of virulence (2). The coronaviruses differ from these other classes of viruses in several ways. Cleavage of the spike protein of porcine transmissible gastroenteritis virus and feline infectious peritonitis virus does not occur. Since these viruses cause fusion of infected cells, it is clear that cleavage of S is not a strict requirement for fusion for all coronaviruses. While cleavage of the paramyxovirus, myxovirus, and retrovirus fusion proteins exposes the hydrophobic fusion peptide at the newly exposed amino terminus, this is not true of the cleaved coronavirus spike protein. A hydrophobic fusion peptide has not yet been identified for any of the coronaviruses. Furthermore, the mutants described here do not cleave the spike protein, but are still infectious in cultured cells and cause a significant amount of cell-to-cell fusion at late times after infection. Finally, virulence and organ tropism are not linked to cleavage in this group of MHV-A59 fusion mutants (unpublished data).

Cellular proteases have been identified that specifically cleave proteins in regions of basic amino acids, and it has been suggested that they may be responsible for the cleavage of viral envelope glycoproteins (10). It is likely that these or similar proteases are involved in the processing of the MHV spike protein. It is tempting to speculate that the absence of such a protease in astrocytes could remove the selection to maintain the cleavage signal in the spike genes, assuming that efficient cell fusion does provide a growth advantage to MHV in other cell types. We are investigating this possibility

by examining the structure of S in virions purified from infected astrocytes.

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